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Ancestral variation and the potential for genetic accommodation in larval amphibians: implications for the evolution of novel feeding strategies

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SUMMARY Few studies provide empirical evidence for phenotypic plasticity's role in the evolution of novel traits. One way to do so is to test whether latent plasticity is present in an ancestor that can be refined, enhanced, or diminished by selection in derived taxa (through "genetic accommodation"), thereby producing novel traits. Here, we evaluated whether gut plasticity preceded and promoted the evolution of a novel feeding strategy in spadefoot toad tadpoles. We studied *Scaphiopus couchii*, whose tadpoles develop an elongate gut and consume only detritus, and two derived species, *Spea multiplicata* and *Sp. bombifrons*, whose tadpoles also express a novel, short-gut phenotype in response to a novel resource (anostracan shrimp). Consistent with the expectations of plasticity-mediated trait evolution, we found that shrimp induced a range of phenotypes in *Scaphiopus* that were not produced with detritus. This plasticity was either suppressed or exaggerated in *Spea* depending on whether the induced phenotypes were adaptive. Moreover, in contrast to its effects on morphology, shrimp induced little or no functional plasticity, as assessed by gut cell proliferation, in *Scaphiopus*. Shrimp did, however, induce substantial proliferation in *Sp. bombifrons*, the species that consumes the most shrimp and that produces the short-gut phenotype the most frequently. Thus, if *Spea* had ancestral morphological plasticity in response to a novel diet, their shrimp-induced short-gut morphology may have undergone subsequent genetic accommodation that improved its functionality. Hence, diet-induced phenotypic plasticity may have preceded and even promoted the evolution of a novel phenotype.

INTRODUCTION

One of biology's most significant unresolved issues is to understand how novel, complex phenotypes originate, both developmentally and evolutionarily. Long-standing theory suggests that new traits may begin as environmentally initiated phenotypic change (Baldwin 1896; Schmalhausen 1949; Waddington 1959; West-Eberhard 2003). Consider that most organisms have the capacity to alter their phenotype in response to external stimuli through the process of phenotypic plasticity (reviewed in West-Eberhard 2003). According to the theory, some such environmentally triggered variants may, by chance, improve an organism's viability under stressful conditions (Baldwin 1896; Schmalhausen 1949). If heritable variation exists among members of a population in their tendency to produce the newly favored trait, then selection should favor those alleles or gene combinations that best stabilize, refine, and extend the new trait's expression (a process known as "genetic accommodation," West-Eberhard 2003). Over evolutionary time, a trait that was initially produced in response to an environmental stimulus may eventually become either canalized or become part of an alternate phenotype controlled by a developmental switch (polyphenism). In this way, novel traits may emerge from an organism's flexible developmental system.

Here, we explore phenotypic plasticity's role in the origin of novel resource-acquisition traits. In particular, we examine whether and how phenotypic plasticity mediates the evolutionary transition to a novel diet. The focus of our research is the origin of a short, carnivore-like gut found in certain larval amphibians. Amphibians, like many other vertebrate species, show pronounced variation in diet and gut morphology, both within and between species (Piersma and Lindstrom 1997; Sabat et al. 1998; Hume et al. 2002; Secor et al. 2002; Starck and Rahmaan 2003; Starck and Wang 2005). In some cases, dietary signals induce a change in gut morphology through phenotypic plasticity: ingestion of small particles of plant material induces an elongate gut (observed in most amphibian larvae, Altig et al. 2007), whereas large particles of animal prey induce a greatly shortened gut (Yung 1904; Babak 1905). These morphologies are thought to be adaptive for assimilating low and high-nutrient diets, respectively (Barton and Houston 1993; Hume 2005). Such plasticity is expected in the gut because, although it is generally the most expensive organ

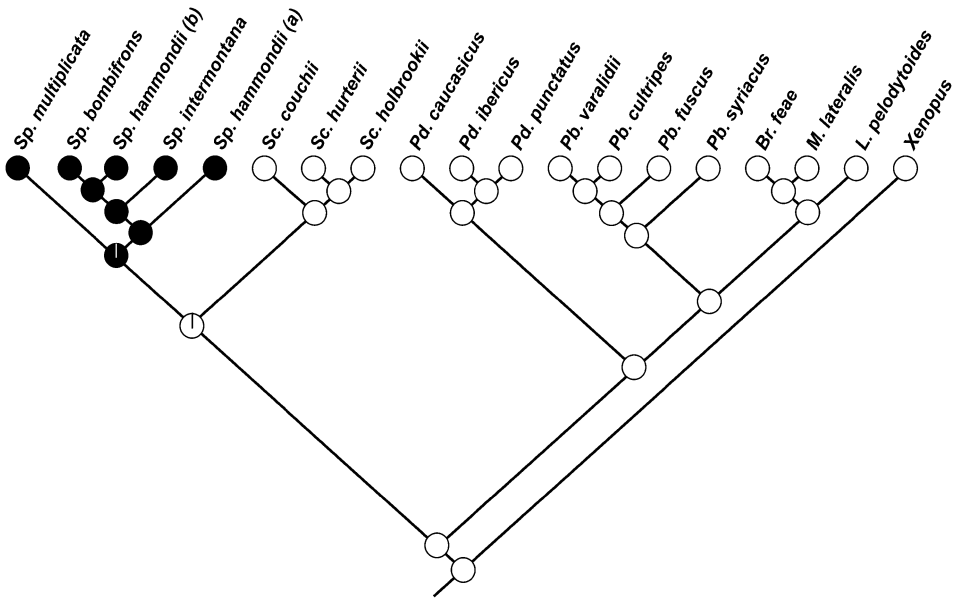

Fig. 1. Relative support for omnivory (white) and facultative carnivory (black) in the ancestors of Pelobatoidea and outgroup, *Xenopus*. Transition rates are equal ($\hat{q} = 0.03$, the rate estimated by ML under the Mk1 model) and branch lengths are all equal to one. The tree is derived from Fig. 1 of García-París et al. (2003). Genera abbreviations: *Sp.*, *Spea*; *Sc.*, *Scaphiopus*; *Pd.*, *Pelodytes*; *Pb.*, *Pelobates*; *Br.*, *Brachytarsophrys*; *M.*, *Megophrys*; and *L.*, *Leptolalax*.

to generate and maintain, it also dictates an individual's net energy gain from a given meal (Hume 2005). In effect, expressing the inappropriate gut morphology or physiology for a given diet may place an individual at an extreme selective disadvantage (Diamond 1991).

We were specifically interested in determining whether intraspecific plasticity forms the basis for the evolution of interspecific differences in gut development and resource use. Our study focuses on spadefoot toad tadpoles. During their larval stage, spadefoot toad species vary in their ability to consume an alternate diet. Specifically, tadpoles in the genus *Spea* may depart from their usual diet of minute organic material and prey on large invertebrates (anostracan fairy shrimp) and other tadpoles (Pomeroy 1981; Pfennig 1990). Moreover, where they co-occur in southeastern Arizona, *Sp. bombifrons* and *Sp. multiplicata* have diverged in feeding behavior, with *Sp. bombifrons* possessing the higher inherent propensity to consume shrimp (Pfennig and Murphy 2000; Pfennig and Murphy 2002). Competition for shrimp may be so intense that sympatric *Sp. multiplicata* (the inferior competitor for shrimp) specializes only on detritus (Pfennig and Murphy 2000). Unlike *Spea*, a third species of spadefoot toad inhabiting southeastern Arizona—*Scaphiopus couchii*—feeds exclusively on minute organic material (detritus; D. Pfennig, personal observation). Because the larvae of *Scaphiopus* (*Spea*'s sister genus García-París et al. 2003), and most other anuran species feed on detritus and microorganisms (Altig et al. 2007), it is likely that the ancestors of *Spea* shared the same omnivorous feeding strategy as *Sc. couchii*, and that carnivory in this genus is a derived trait (confirmed in the present study; see "Results" and Fig. 1). Moreover, different feeding strategies are associated with different morphologies. In contrast to the long, coiled gut of most anuran larvae (including *Scaphiopus*), *Spea* larvae that consume shrimp facultatively develop a relatively short, uncoiled gut. It has not been observed, however, whether *Sc. couchii* possess the same morphological response when they are forced to consume shrimp. Further, it is unknown whether *Sc. couchii* has the ability to assimilate this potentially high-quality resource in the same manner as *Spea*.

In this study, we focused on the above three species of spadefoot toads to evaluate the possible role of genetic accommodation in morphological evolution. Documenting accommodation in natural populations is difficult because once a trait or reaction norm has spread through a population, it is impossible to distinguish whether it arose in response to an environmental stimulus or through selection on standing phenotypic variation (Hall 2001; Moczek 2007). Nonetheless, there are specific predictions that, if met, would provide convincing evidence that plasticity played a significant role in trait evolution.

First, ancestral species (or closely-related species that share the ancestral condition), when challenged with a novel environmental stimulus (i.e., a stimulus experienced by species with derived traits), should exhibit plasticity in the trait of interest (Badyaev et al. 2005; West-Eberhard 2005; Ghalambor et al. 2007). Finding such a pattern would suggest that "hidden" sensitivity to environmental stimuli existed in the ancestor that could have provided the variation on which natural selection could act to promote the evolution of novel phenotypes in derived taxa (Rutherford and Lindquist 1998; Hall 2001; Queitsch et al. 2002; Sangster et al. 2004; Badyaev et al. 2005; West-Eberhard 2005). Second, because no known mechanism can generate adaptive variation in anticipation of a novel environmental stimulus (Moczek 2007), such phenotypic variation should be random with respect to its adaptive

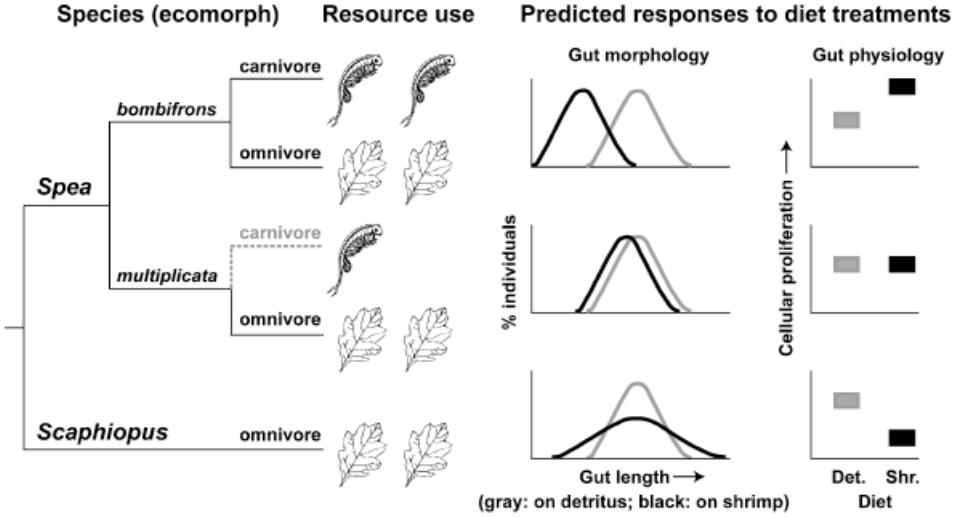

Fig. 2. The predicted responses of three spadefoot toad species to shrimp (shr.) and detritus (det.) if genetic accommodation played a role in the evolution of *Spea*'s feeding strategies. *Scaphiopus* is omnivorous and uses only one resource, detritus (indicated by leaves), whereas *Spea* has evolved facultative carnivory and can use detritus or shrimp. If developmental plasticity preceded the evolution of carnivory in *Spea*, we would expect *Scaphiopus* to exhibit morphological variation in response to shrimp, but a weak functional response (as assayed by gut cellular proliferation). We would expect species of *Spea* to have developed diet-induced polyphenism in environments where they can exploit both resources (as is *Sp. bombifrons*), or to have canalized a particular morphology in environments where they specialize on only one resource (as in sympatric *Sp. multiplicata*). Moreover, because modern species of *Spea* have an evolutionary history of consuming shrimp, they should also elicit a functional response to this resource.

value in the inducing environment. Further, we would also expect the magnitude of the plastic response to vary between genotypic backgrounds; e.g., across different sibships (Waddington 1959; Queitsch et al. 2002).

To determine whether phenotypic plasticity may have facilitated the evolution of novel feeding morphologies in larval spadefoot toads, we examined initial patterns of plasticity (by measuring the response of a species with the ancestral condition on a novel diet) and how that plasticity has evolved under specific diet regimes (by measuring the response of derived species on their current diets). We also determined whether there was elaboration of these traits consistent with ancestral plasticity being modified by selection in descendent species. Specifically, we characterized larval gut plasticity in *Sc. couchii*, *Sp. multiplicata*, and *Sp. bombifrons* 24 h after being fed either detritus or shrimp to test the key prediction that gut plasticity was present before the evolution of novel gut morphologies. We then measured intestinal cellular proliferation across all three species during the time of initial diet-induced divergence to determine whether a healthy physiological response, possibly the result of genetic accommodation, had evolved only in species that recurrently used shrimp. We summarize our predictions in Fig. 2.

MATERIALS AND METHODS

Ancestral character state reconstruction

First, we sought to determine if *Sc. couchii* could serve as an appropriate substitute for *Spea*'s ancestral condition. As noted in "Introduction," the larvae of most anuran species (including those in *Spea*'s sister genus, *Scaphiopus*: García-París et al. 2003) are omnivorous, feeding on detritus and microorganisms (Altig et al. 2007). We therefore explored whether the ancestors of *Spea* likely shared the same omnivorous feeding strategy as *Sc. couchii* and whether carnivory is a derived trait in *Spea* (where carnivory is defined as the ability to capture and consume living, macroscopic prey, such as anostracan shrimp and other tadpoles).

To address this issue, we used a phylogenetic framework to study the evolution of carnivory in larval anurans. Different feeding strategies (i.e., omnivory and carnivory) are associated with different morphologies. Most anuran larvae express a phenotype characterized by smooth keratinized mouthparts, small jaw muscles, and an elongate gut. We refer to this phenotype hereafter as the "omnivore" ecomorph. In contrast, *Spea* larvae, when fed live shrimp, may facultatively express a phenotype characterized by notched and serrated keratinized mouthparts, large jaw muscles, and a short gut. We refer to this phenotype hereafter as the "carnivore" ecomorph (this ecomorph has been documented in all four *Spea* species: *Sp. bombifrons* and *Sp. multiplicata*: Pomeroy (1981); *Sp. intermontana*: Hall (1998); *Sp. hammondii*: J. Arendt, pers. comm.). Thus, we used the presence (or absence) of the distinctive carnivore phenotype to indicate whether a particular clade had (or had not) evolved carnivory.

To determine if carnivory is a derived trait in *Spea*, we used the maximum likelihood (ML) model Mk1 in Mesquite 2.01 (Maddison and Maddison 2007) to reconstruct *Spea*'s ancestral feeding strategy. We used a tree based on the analysis of García-París et al. (2003), which was constructed using ML and Bayesian analyses of partial sequences of two mitochondrial genes (cytochrome *b* and 16S RNA). Branches of the same species were consolidated when they were monophyletic. Each species was assigned a feeding strategy (carnivory or omnivory) based on whether or not the carnivore ecomorph had been documented in that particular species. Species that express solely an omnivore ecomorph were given character state "0," whereas those that have the ability to express an alternate carnivore ecomorph were given

character state "1." All transition rates were assumed equal, and all branch lengths were set to one. The best character state for a given node was determined by whether the log likelihoods of carnivory and omnivory differed by 2 (the decision threshold); if the log likelihoods of the two states differed by 2 or more, the state with the lower likelihood was rejected (Maddison and Maddison 2007).

Breedings

All breedings were performed using pairs of animals captured near Portal, Arizona, USA. Moreover, the *Sp. multiplicata* used in the experiments below were derived from populations that are sympatric with *Sp. bombifrons*. Thus, based on earlier work (Pfennig and Murphy 2000; Pfennig and Murphy 2002), we expected these *Sp. multiplicata* to be canalized for omnivore production (confirmed in this study; see "Results"). Captured animals had been housed in a colony at the University of North Carolina, Chapel Hill for 1–2 years. To induce breeding, adults were injected with 0.07 ml Lh-Rh luteinizing hormone (Sigma number L-7134) and left overnight in nursery tanks. All procedures were carried out in compliance with the Institutional Animal Care and Use Committee at the University of North Carolina, Chapel Hill, under application # 03-0110.

Characterizing gut morphogenesis on alternative diets

To infer whether morphological gut plasticity was likely present in ancestral *Spea*, we examined the gut morphology of larvae from 2 to 3 sibships of *Sp. bombifrons*, *Sp. multiplicata*, and *Sc. couchii* after being fed fairy shrimp or detritus. After animals had bred, 192 larvae from each sibship were transferred to individual 3 oz plastic cups that were randomized and interspersed on racks in the same room maintained at 26°C and on a 14 h L:10 h D light cycle. Four days after breeding, each larva was fed either 0.25 ml (approximately 50 mg wet weight, approximately 10 mg dry weight) of brine shrimp nauplii or 10 mg of ground fish food; brine shrimp resemble the fairy shrimp that *Spea* feed on in nature, whereas ground fish food resembles detritus. Twenty-four hours later, tadpoles that had cleared their cups of food (ensuring that all individuals—even *Sc. couchii* in the shrimp treatment—had consumed the same amount of diet) were euthanized with tricaine methosulfonate (MS 222), fixed in 10% formalin and stored in 70% ethanol. Only those that cleared their cups of food (family average for shrimp/detritus = 72.5/79 for *Sp. bombifrons*, 63.3/77.6 for *Sp. multiplicata*, and 56.5/94.5 for *Sc. couchii*) were used in subsequent analyses.

To assist in visualizing gut morphology, the ventral skin of each preserved tadpole was removed before photographing the guts with a Leica (Wetzlar, Germany) DFC480 R2 Camera (magnification, × 2.5). Gut morphology was characterized by the number of revolutions the gut had completed (partial revolutions were estimated to one-tenth of a full revolution, e.g., 2.7 revolutions). We used three methods to determine the patterns of diet-induced gut morphologies among sibships and species. First, we used a nonparametric Wilcoxon's rank-sum test to compare the number of gut revolutions induced by detritus or shrimp in each sibship. A nonparametric analysis was used because the data failed to meet the assumption of normality, even after transformation. Second, we used Levene's test to compare how variable diet-induced phenotypes were in each sibship. Finally, we determined how frequently extreme morphologies occurred in response to diet by assessing outliers. Outliers in each sibship fell above and below the upper fences of the sibship-specific (pooled-treatments) distributions of revolutions (upper fence = upper quartile value + 1.5 × interquartile range, lower fence = lower quartile value − 1.5 × interquartile range). All statistical analyses were performed with JMP version 5.1.2 Statistical Software (SAS Institute, Cary, NC, USA).

Measuring cellular proliferation

To determine whether morphologies induced by shrimp in ancestral *Spea* have undergone modification in derived species that routinely consume this diet, we measured cellular proliferation in the larval intestines of each spadefoot species during the initial time of diet-induced morphological divergence. Following the consumption of a meal, intestinal cellular proliferation may indicate how well an individual assimilates a particular diet (e.g., see Rebel et al. 2006; see also the "Discussion"). We therefore measured intestinal cellular proliferation, as indicated by the number of cells undergoing mitosis, in each sibship and species.

We obtained embryos from two sibships each of *Sp. bombifrons*, *Sp. multiplicata*, and *Sc. couchii* (all sibships were different from those breedings used in characterizing gut morphology). One hundred forty-four larvae from each sibship were transferred to individual 3 oz plastic cups and fed either 0.25 ml (approximately 50 mg wet weight, approximately 10 mg dry weight) of brine shrimp nauplii, 10 mg of ground fish food, or were given no food. Twenty-four hours later, those that had cleared their cups of food (ensuring that all individuals—even *Sc. couchii* in the shrimp treatment—had consumed the same amount of diet) were anesthetized with 5% MS 222 and fixed for immunohistochemistry (IHC) following the protocol of (Langer 2004). A dilute (1:100) polyclonal antibody, anti-phosphohistone H3 (Upstate USA—Millipore, Billerica, MA, USA), was added to the samples and left to incubate at 4°C overnight. After the incubation, the samples were left at room temperature for an hour before washing with a phosphate buffer and 0.1% Tween-20 solution (PBT) for three 2 h washes. The PBT was removed and a dilute (1:400) Alexa Fluor 568 labeled goat-anti-rabbit secondary antibody was added to the samples with blocking solution for an overnight incubation at 4°C. The secondary antibody was removed by three 2 h washes with PBT. Specimens were mounted on slides in antifade mounting medium with 4′,6-diamidino-2-phenylindole (DAPI, for the identification of cell nuclei) (Invitrogen, Carlsbad, CA, USA). Images of guts were stained for IHC, viewed on a Leica DM 5000B microscope (magnification × 3.8), and captured using the Simple PCI imaging system (Sewickley, PA, USA). We used an approximately 0.5 mm × 0.5 mm section in the anterior gut (immediately posterior to the stomach) to quantify proliferation; absorption of nutrients in anuran larval gut is know to decline anterioposteriorly (Toloza and Diamond 1990; Ishizuya-Oka et al. 1997) and it is more likely that the anterior portion of larval intestines respond morphologically and functionally to luminal nutrients (Secor 2005; Tappenden 2006). Mitotic cells in this area were identified by their intense staining (red) against a field of DAPI stained cells (blue), counted, and divided by the area of interest to create an index of cellular response. All areas were determined using NIH ImageJ (Rasband 1997–2006).

To assess differential cellular responses among species and sibships, a nested three-way model was used with mitotic cells per unit area as the dependent variable, and sibship nested within species, species, diet, and interactions as factors. Because a significant species by diet interaction was detected (ANOVA, $F = 12.73$, $P < 0.0001$), the effect of diet on each species was analyzed separately using a two-way ANOVA (with diet and sibship as factors). A sibship × diet effect was also detected in *Sp. bombifrons* (ANOVA, $F = 7.89$, $P = 0.0011$), so each sibship within that species was analyzed with a one-way ANOVA. When significant diet effects were found, one-tailed post hoc Bonferroni's *t* tests were performed between diets using $\alpha = 0.05$ (where it was predicted that the response to shrimp > response to detritus, and the response to detritus > unfed response). As before, all statistical analyses were performed with JMP version 5.1.2 Statistical Software (SAS Institute, Cary, NC, USA).

RESULTS

Ancestral character state reconstruction

The ML reconstruction suggested that *Spea*'s ancestral feeding strategy was likely omnivory (Fig. 1). The ancestral node that gave rise to Schapiopodidae (i.e., the clade containing genera *Scaphiopus* and *Spea*) favored omnivory as the best character state (relative likelihoods for omnivory:carnivory were 7.3:1).

Characterizing gut morphogenesis on alternative diets

Spadefoot species and sibships differed in their ability to express the short-gut morphology when fed shrimp. In *Sc. couchii,* the species that does not feed on shrimp in nature, shrimp failed to induce shorter guts on average in both sibships (mean gut revolutions ± SD for sibship one: 1.83 ± 0.4 on shrimp, 1.90 ± 0.4 on detritus, Wilcoxon's rank-sum, $S = 3301$, $P = 0.38$; for sibship two: 2.14 ± 0.4 on shrimp, 2.25 ± 0.3 on detritus, Wilcoxon's rank-sum, $S = 4659$, $P = 0.08$). However, when fed shrimp, both *Sc. couchii* sibships produced a wider range of phenotypes than when fed detritus (range of gut revolutions for sibship one: 0.9–3.1 on shrimp, 1.3–3.0 on detritus; for sibship two: 1.0–3.2 on shrimp, 1.6–3.1 on detritus) and, in sibship two, more variable (Levene's test, $P = 0.05$) and extreme phenotypes (Figs. 3 and 4). For *Sp. multiplicata*, the species that has historically experienced shrimp, but has more recently experienced competitive exclusion from this resource, we found that shrimp induced shorter guts (1.75 ± 0.4) than detritus (2.80 ± 0.4) in only one of three sibships (Wilcoxon's rank-sum, $S = 7844$, $P < 0.0001$). None of the *Sp. multiplicata* sibships responded to shrimp by producing a wider range of gut phenotypes or greater variance in gut morphology and, across all three sibships, shrimp generated only one extreme morphology (the one long outlier in Fig. 4). Finally, in *Sp. bombifrons*, the species that has the highest propensity to consume this resource, shrimp produced shorter gut morphologies on average in both sibships (for sibship one: 1.88 ± 0.5 on shrimp, 2.20 ± 0.4 on detritus, Wilcoxon's rank-sum, $S = 4215$, $P < 0.0001$; for sibship two: 2.27 ± 0.4 on shrimp, 2.53 ± 0.04 on detritus, Wilcoxon's rank-sum, $S = 4757$, $P = 0.0001$), and also generated the greatest number of extremely short-gut morphologies (10 short

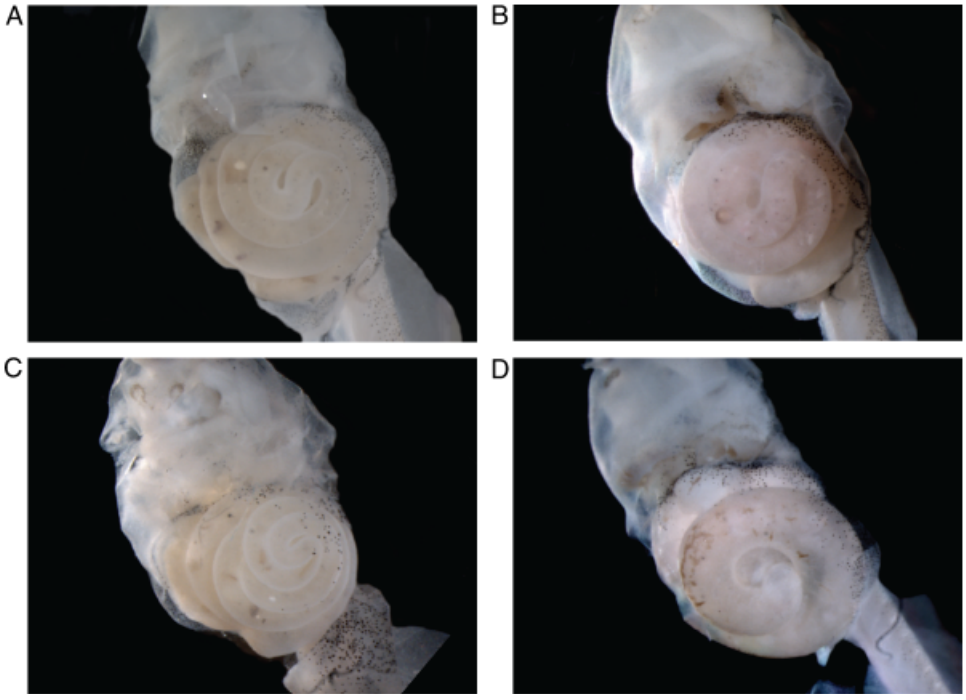

Fig. 3. (A, B, C, and D) Different diets contribute to the development of different morphologies in *Sp. bombifrons* at 24 h-post feeding. (A) A detritus-fed individual that has intermediate gut morphology. (B) A shrimp-fed individual that has intermediate gut morphology. (C) A detritus-fed individual that has an extreme, long-gut morphology. (D) A shrimp-fed individual that has an extreme, short-gut morphology. Extreme morphologies observed in other spadefoot species were similar to the morphologies depicted here. Image backgrounds were made uniform with Adobe Photoshop CS2 (9.0).

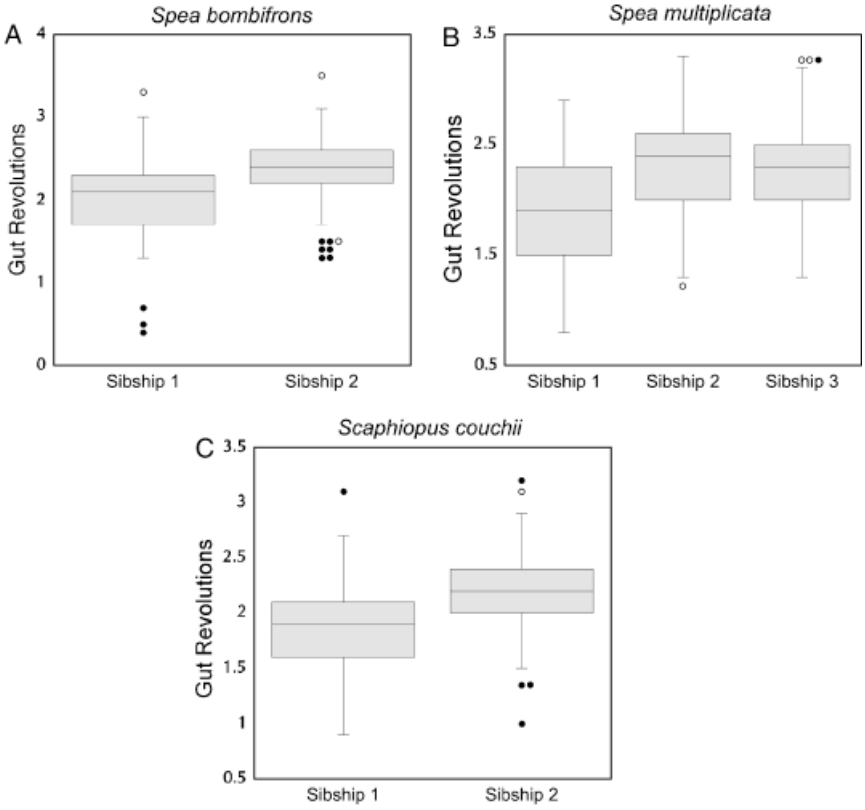

Fig. 4. (A, B, and C) Distributions of extreme morphologies (outliers) across species. Lines within boxes are sibship-specific (pooled-treatments; i.e., both diet treatments combined) mean morphologies, where extreme morphologies (outliers) are represented as circles (points greater than UQ+1.5 × IQD or less than LQ − 1.5 × IQD, where UQ, upper quartile; LQ, lower quartile, and IQD, inter quartile distance). Closed and open circles indicate shrimp and detritus-induced morphologies, respectively. Gut morphologies become short with fewer gut revolutions (e.g., in B, sibship 2 has one extreme "short-gut" morphology).

outliers, Figs. 3 and 4). Shrimp-fed *Sp. bombifrons* did not exhibit more variable gut phenotypes than those fed detritus.

MEASURING CELLULAR PROLIFERATION

Diet influenced cellular proliferation in the anterior region of the guts of all three spadefoot species (Figs. 5 and 6). In *Sc. couchii*, detritus (mean ± SD: 597.14 ± 40.7 mitotic cells/mm^2) had a significantly greater effect than both the shrimp (313.42 ± 34.8 mitotic cells/mm^2; $t = 5.29$, $P < 0.0001$, one-tailed Bonferroni's t test for here and all comparisons below) and unfed treatment (242.78 ± 37.9 mitotic cells/mm^2; $t = 6.37$, $P < 0.0001$), but the shrimp and unfed treatment were not significantly different from each other ($t = 1.37$, $P = 0.09$). In *Sp. multiplicata*, the effects of shrimp (664.94 ± 53.1 mitotic cells/mm^2) and detritus (614.73 ± 50.0 mitotic cells/mm^2) were not significantly different from each other ($t = 0.75$, $P = 0.23$), but both were significantly greater than the unfed treatment (371.60 ± 51.9 mitotic cells/mm^2; shrimp

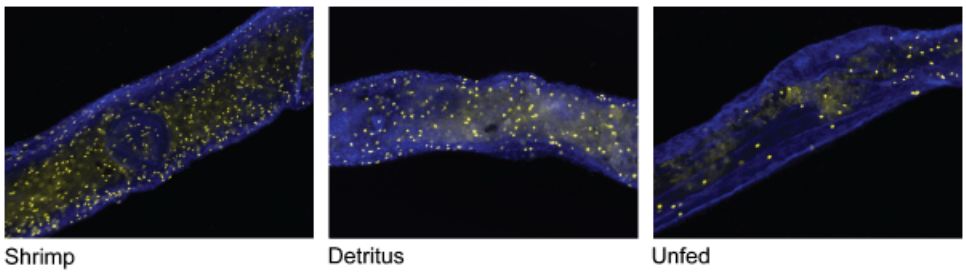

Fig. 5. Proliferation in the anterior intestine of *Sp. bombifrons* in response to a shrimp, detritus and unfed treatment, demonstrating intense, moderate, and weak mitotic activity, respectively (magnification × 3.8; images were enlarged with Adobe Photoshop to a final magnification of × 5.4). Spadefoot intestines were subject to whole mount immunohistochemical staining with an anti-phosphohistone antibody, visualized with an Alexa Red conjugated secondary antibody (fluorescence is pseudocolored yellow for contrast). The punctate staining indicates nuclei undergoing mitosis; the more diffuse yellow staining is background due to trapped gut contents. Nuclei of all cells are counterstained with DAPI (blue). Image backgrounds were made uniform with Adobe Photoshop.

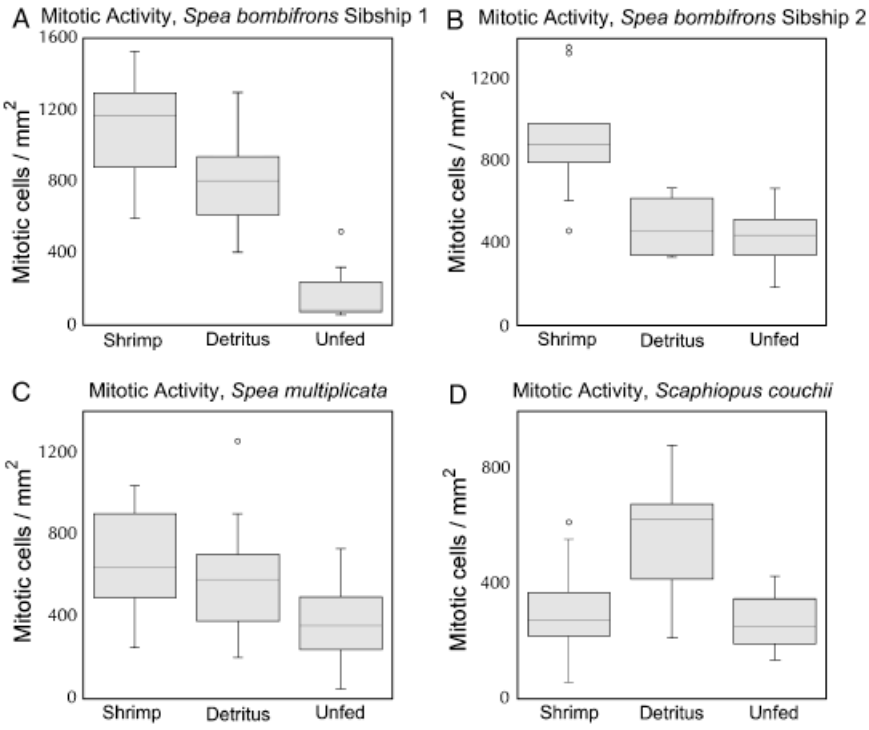

Fig. 6. (A, B, C, and D) Distributions of cellular proliferation in the anterior intestines of three species of spadefoot toads when fed different diets. Lines within boxes indicate a group's median value and circles indicate outliers. The lines extending from the top and bottom of each box represent the maximum and minimum values within the data set that fall within an acceptable range. (A, B) Two sibships of *Sp. bombifrons*. Sibship 1 experienced more proliferation when fed shrimp than when fed detritus ($P = 0.02$), and more when fed detritus than when unfed ($P < 0.001$). Sibship 2 experienced more proliferation when fed shrimp than when fed detritus or when unfed ($P < 0.0001$). (C) For two sibships of *Sp. multiplicata* that showed statistically indistinguishable results (family × diet, $P = 0.59$), the response to shrimp and detritus was similar ($P = 0.25$) and both responses were greater than when unfed ($P < 0.0007$). (D) For two sibships of *Sc. couchii* that showed statistically indistinguishable results (family × diet, $P = 0.77$), the response to detritus was greater than its response to shrimp or being unfed ($P < 0.0001$); the responses to shrimp and to being unfed were not statistically different ($P = 0.08$).

vs. unfed, $t = 3.93$, $P < 0.0001$; detritus vs. unfed, $t = 3.23$, $P = 0.0011$). In the first sibship of *Sp. bombifrons*, shrimp had a significantly greater effect than detritus (for detritus: 1097.73 ± 82.4 mitotic cells/mm^2; for shrimp: 821.80 ± 92.1 mitotic cells/mm^2; $t = 2.31$, $P = 0.015$), and detritus had a significantly greater effect than the unfed treatment (175.68 ± 86.8 mitotic cells/mm^2; $t = 5.23$, $P < 0.0001$). In the second family of *Sp. bombifrons*, shrimp (908.58 ± 61.3 mitotic cells/mm^2) had a significantly greater effect than detritus and no food (for detritus: 480.82 ± 79.2 mitotic cells/mm^2, for unfed: 423.69 ± 56.0 mitotic cells/mm^2; for shrimp vs. detritus, $t = 4.27$, $P < 0.0001$; for shrimp vs. unfed, $t = 5.84$, $P < 0.0001$), but the effects of detritus and being unfed were similar ($t = 0.59$, $P = 0.28$). Thus, these results suggest that shrimp elicits the most cellular proliferation in *Sp. bombifrons*, less proliferation in *Sp. multiplicata*, and the least in *Sc. couchii*.

DISCUSSION

Adopting a new dietary resource can facilitate rapid adaptive evolution in organisms (Reznick and Ghalambor 2001), allowing them to occupy new habitats and shaping their interactions with other species (Karasov and Diamond 1988). Although feeding strategies vary enormously, even among closely related taxa, little is known about how organisms make the transition from an ancestral to a novel diet. One plausible mechanism is that phenotypic accommodation (the induction of novel morphology due to an individual's inherent plasticity) allows an organism to persist under new environmental conditions (West-Eberhard 2003; West-Eberhard 2005; Pigliucci et al. 2006). Subsequently, natural selection may favor genetic combinations that improve and extend the expression and functionality of the novel morphology (a process known as genetic accommodation). Although studies have shown that phenotypic and genetic accommodation can occur in a lab setting (Queitsch et al. 2002; Suzuki and Nijhout 2006), to date, there is a paucity of evidence from natural populations (Hall 2001; Pigliucci and Murren 2003; West-Eberhard 2005; Braendle and Flatt 2006; Ghalambor et al. 2007; Moczek 2007; for possible examples from nature, see Van Tienderen 1990; Gurevitch 1992; Day et al. 1994; Losos et al. 2000; Pfennig and Murphy 2002; Sword 2002; Gomez-Mestre and Buchholz 2006; Parsons and Robinson 2006).

For traits to evolve by genetic accommodation, an ancestral lineage must already harbor underlying genetic variation that can be exposed by either a sensitizing mutation or environmental stimulus (Sangster et al. 2004; Suzuki and Nijhout 2006). Such "hidden" genetic variation is neutral until the genetic or environmental stimulus causes it to be expressed phenotypically and thereby exposed to selection (Bergman and Siegal 2003; Hermisson and Wagner 2004). This process often increases the range of phenotypes normally exhibited by

an individual (Schlichting and Smith 2002; Sangster et al. 2004; Badyaev 2005; Badyaev et al. 2005). If an exposed phenotype is adaptive in a given environment, accommodation may cause the population to evolve a developmental switch point for multiple traits (environmental polyphenism), or become canalized for one trait (genetic assimilation, e.g., see Suzuki and Nijhout 2006). Thus, if *Sc. couchii* is an acceptable model for *Spea*'s ancestral condition (as our data suggest that it is; Fig. 1), then we would expect *Sc. couchii* to exhibit morphological gut plasticity in response to consuming shrimp, and we would expect contemporary species of *Spea* either to have developed diet-induced polyphenism in environments where they can exploit both resources, or to have canalized a particular morphology in environments where they specialize on only one resource (e.g., see Fig. 2).

Ancestral *Spea* were most likely similar to *Sc. couchii* in digestive morphology and physiology: An ancestral character state reconstruction revealed that *Spea*'s ancestral feeding strategy was likely omnivory (Fig. 1), the strategy exhibited by *Sc. couchii*. Moreover, our measure of cellular proliferation in larval intestines indicates that *Sc. couchii* assimilates detritus with greater efficiency than shrimp. Thus, shrimp may have been, at least initially, a suboptimal component of the ancestral *Spea* species' diet. Nonetheless, *Sc. couchii* can survive for at least 9 days post-fertilization when fed solely shrimp (unpublished data), indicating that ancestral *Spea* could also have survived on this novel diet. The ability to persist on shrimp would have allowed preexisting variation for larval morphological gut plasticity to be selected upon in subsequent generations. Our measures of morphological plasticity in *Sc. couchii* suggest that ancestral *Spea* populations likely harbored variation in their ability to respond to shrimp, and this ability varied between sibships. Indeed, in both *Sc. couchii* sibships, the range of phenotypes expressed by shrimp-fed larvae was greater than the range of phenotypes expressed by detritus-fed larvae (as we had predicted; see Fig. 2). Moreover, also as predicted (see "Introduction"), sibships differed in how responsive they were to shrimp and how frequently they expressed extreme morphologies.

Because *Sp. bombifrons* outcompetes *Sp. multiplicata* for shrimp (Pfennig and Murphy 2000), sympatric *Sp. bombifrons* and *Sp. multiplicata* have differential access to shrimp, even in the same ponds. Thus, we would expect genetic accommodation to have produced different feeding strategies in these two species (Fig. 2). *Sp. bombifrons* most likely benefits from retaining its ancestral plasticity; the availability of shrimp varies spatially (between ponds), temporally (between years), and even within their larval period (in some ponds, shrimp are eliminated before the tadpoles metamorphose; D. Pfennig, personal observation). This variability in resource abundance both within and between generations should favor the evolution of within-generational plasticity (West-Eberhard 2003; Young and Badyaev 2007). Thus, although shrimp is a more nutritious resource for *Sp. bombifrons* (Pfennig 2000; Pfennig and Murphy 2000), evolving a developmental switch for the short-gut morphology would allow it to reap benefits from multiple environmental conditions. Unlike *Sc. couchii*, that produced both long and short outliers when fed shrimp, *Sp. bombifrons* only produced short outliers (i.e., extremely short guts) when fed shrimp, and produced mostly long outliers when fed detritus. *Sp. bombifrons'* tendency to produce the "correct" morphologies in response to shrimp and detritus probably reflects its fluctuating history with both resources.

In contrast, *Sp. multiplicata* larvae found in sympatry with *Sp. bombifrons* (i.e., the population used in this experiment) are poor competitors for shrimp, *even when it is available* (Pfennig and Murphy 2000; Pfennig and Murphy 2002). Thus, *Sp. multiplicata* larvae would benefit from having a canalized, long-gut morphology that is better suited for foraging on detritus (the majority of its diet). Because sympatric *Sp. multiplicata* may encounter and occasionally consume shrimp, they may have, early in their evolutionary history, been prone to producing a shorter gut morphology. However, because this short gut morphology would have been maladaptive in sympatric *S. multiplicata* (because they are inferior shrimp competitors: Pfennig and Murphy 2000), genetic combinations that stabilized the long-gut morphology should have been selectively favored. Indeed, as expected (Fig. 2), *Sp. multiplicata* never produced short outliers in response to shrimp, and shrimp only shortened average gut morphology in one of three sibships tested. Thus, sympatric *Sp. multiplicata* populations have apparently stabilized the long-gut phenotype by genetic compensation (accommodation that restores the original phenotype, Grether 2005) with respect to *Spea*'s ancestral phenotype, or secondarily by genetic assimilation with respect to allopatric *Sp. multiplicata* populations. It is possible that *Sp. multiplicata* exhibits intestinal plasticity with greater frequency in allopatry, where it is the only spadefoot species consuming shrimp, but that comparison has not yet been made.

Not only has the short-gut morphology become more predictably plastic in *Sp. bombifrons,* it has been accompanied by physiological plasticity. Although both diets caused proliferation in the guts of the two *Spea* species, shrimp promoted greater proliferation than detritus in *Sp. bombifrons,* the species that has the greatest propensity to eat shrimp (as predicted; Fig. 2). In contrast, *Sc. couchii*'s response to shrimp was not significantly different from its unfed response (Fig. 6). The physiological and developmental significance of this differential cell proliferation in spadefoots has yet to be determined. In one study, 14-day-old *Gallus* chicks of mothers fed a high-nutrient diet experienced greater intestinal proliferation compared with the offspring of mothers fed a low-nutrient diet (Rebel et al. 2006). The proliferation was concurrent with the expression of genes related to intestinal development, utilization of lipids and nutritional absorption.

Intestinal proliferation in *Spea* larvae may similarly represent a physiological response to a high-nutrient diet.

Because stress responses in organisms are often integrated into one or more endocrinological axes, *Sc. couchii*'s intestinal response to an unfamiliar diet may be mediated by hormonal control. For instance, generalized stress (e.g., declining water availability, increasing conspecific density, food deprivation) activates the hypothalamic–pituitary–interrenal axis in spadefoot toad tadpoles (*Sp. hammondii*), elevating whole-body corticosteroid content that can affect peripheral tissues (Denver 1999; Boorse and Denver 2003). Corticoid-releasing hormone also stimulates the production of thyroid hormone (Denver 1999), which accelerates differentiation and inhibits growth (Boorse and Denver 2003). Moreover, thyroid hormone induces the expression of bone morphogenetic protein 4 (BMP-4), which inhibits proliferation in the larval intestine (Ishizuya-Oka et al. 2006). Thus, if the stress caused by consuming a novel diet resulted in the upregulation of certain hormones in these developing tadpoles, proliferation in the anterior intestine could be significantly reduced. Over time, selection could work directly on these hormones or their target tissues, so that populations routinely consuming shrimp undergo normal gut development (as in derived *Spea*). However, because the relationship between hormones and their effects on tissues are generally complex and stage-dependent (Crespi and Denver 2005), determining the specific role of stress on gut development in spadefoot tadpoles would warrant rigorous investigation.

If *Spea*'s ancestors could not utilize shrimp initially, why did the short-gut morphology become prevalent? In habitats where ancestral *Spea* tadpoles occurred, tadpoles may have found themselves in drying ponds without vegetation or microbial growth, in which shrimp were the only resource (e.g., as in modern "playa" lakes; see Pfennig et al. 2006). In such stressful settings, ancestral *Spea* tadpoles may have been faced with preying on shrimp and other tadpoles to survive. Although ancestral *Spea* may have initially suffered a fitness reduction from this diet, individuals able to induce a shorter gut in this new environment would still have had an evolutionary advantage over individuals that lacked plasticity: Producing a shorter gut would have conserved developmental resources that would be otherwise squandered on an "unemployed" organ (Diamond 1991). Over time, those individuals that were most able to accommodate this change would have been selectively favored (e.g., through expression of appropriate enzymes, nutrient transporters, or hormones).

Further investigation is needed to illuminate the type of genetic variation that was selected on for *Spea* to adopt carnivory as an alternate feeding strategy. For now, the morphological plasticity demonstrated by *Spea*'s sister genus, *Scaphiopus*, suggests that phenotypic plasticity may have played a key role in *Spea*'s ability to persist on a novel resource, and may have even instigated morphological and physiological evolution through genetic accommodation.

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